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Characterization of MC4R Regulation of the Kir7.1 Channel Using the Tl^+ Flux Assay

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Abstract

The family of inward rectifying potassium channels (Kir channels) plays crucial roles in the regulation of heart rhythms, renal excretion, insulin release, and neuronal activity. Their dysfunction has been attributed to numerous diseases such as cardiac arrhythmia, kidney failure and electrolyte imbalance, diabetes mellitus, epilepsy, retinal degeneration, and other neuronal disorders. We have recently demonstrated that the melanocortin-4 receptor (MC4R), a $G\alpha_s$-coupled GPCR, regulates Kir7.1 activity through a mechanism independent of $G\alpha_s$ and cAMP. In contrast to the many other members of the Kir channel family, less is known about the biophysical properties, regulation, and physiological functions of Kir7.1. In addition to using conventional patch clamp techniques, we have employed a high-throughput Tl^+ flux assay to further investigate the kinetics of MC4R-Kir7.1 signaling in vitro. Here, we discuss the employment of the Tl^+ flux assay to study MC4R -mediated regulation of Kir7.1 activity and to screen compounds for drug discovery.

Keywords

Melanocortin 4 receptor (MC4R); Inward rectifying K^+ channels (Kir7.1); Thallium flux assay; Intracellular signaling; High-throughput screening

1 Introduction

Inward rectifying K^+ currents (Kir) were first identified more than a half a century ago [1]. Since their discovery, seven subfamilies of Kir channels have been characterized [2]. Kir channels regulate critical cellular parameters including resting membrane potential, action potential duration, and hormone release. Due to their role in regulating these processes, Kir channels are critical for the proper function of cardiac myocytes, neurons, pancreatic β-cells, renal epithelial cells, glia, and epithelial cells. Thus, a better understanding of how Kir channels are regulated holds great potential for future drug discovery.

Invariably, Kir channels are commonly regulated by the signaling lipid PIP_2 that enables channel opening through direct binding [3, 4]. Besides PIP_2, factors that gate Kir channel conductance are unique to each subfamily. For instance, the G protein-coupled inward-rectifying channels (GIRK, Kir3.x) are opened by the βγ subunits of heterotrimeric G proteins [5], while Kir6.2 (K_{ATP}) is sensitive to intracellular ATP levels [6]. Despite

extensive understanding of some Kir channels, the regulatory mechanisms of other Kir channels including Kir7.1 remain largely uncharacterized.

Recently, our group used patch clamp electrophysiology to describe how Kir7.1, a low conductance weakly rectifying Kir sub-type, is regulated by the Melanocortin 4 Receptor (MC4R)—a G_{s}- coupled GPCR [7]. We observed that MC4R activation decreases Kir7.1 opening even in the presence of G_s inhibitors. In order to understand the molecular mechanisms underlying this interaction, we created a heterologous expression system in HEK293 cells and adapted a thallium (Tl^+)-based screening platform for the quantification of channel opening [8].

The use of Tl^+—an equally permeant ion for K^+ channels—and an intracellular Tl^+-sensitive dye enables determination of potassium channel opening in a 384-well format [8]. Cells are loaded with the thallium sensitive dye, exposed to ligands or vehicle for 20 min and given a bolus of thallium Tl^+. The specific techniques below have been employed to characterize MC4R-Kir7.1 signaling and its modulators. However, these techniques can be used to characterize the nature of any Kir-GPCR interaction.

2 Materials

2.1 Assay Buffers

1. Hanks Buffered Salt Solution (HBSS) with Ca^{2+} and Mg^{2+} (Thermo Scientific cat #14025076): 140 mg/L $CaCl_2$, 100 mg/L $MgCl_2{\cdot}6H_2O$, 100 mg/L $MgSO_4{\cdot}7H_2O$, 400 mg/L KCl, 60 mg/L KH_2PO_4, 350 mg/L $NaHCO_3$, 8 g/L NaCl, 48 mg/L Na_2HPO4 (anhydrous), 1 g/L D-Glucose.
2. 1 M HEPES solution: Dissolve 119.15 g HEPES (free acid) in 400 mL ddH_2O. Add 5 M NaOH dropwise to achieve a pH of7.0. Add ddH_2O until the solution reaches a final volume of 500 mL. Filter sterilize.
3. Assay Buffer: Add 10 mL of a 1 M HEPES solution to 500 mL of HBSS w/Ca^{2+} & Mg^{2+}. pH solution to 7.4 with 5 M NaOH.
4. 6.7% w/v pluronic acid solution in DMSO: Dilute 200 mL of a 20% Pluronic acid solution (Thermo Scientific cat #P3000MP) with 400 μL anhydrous DMSO to obtain a 6.7% pluronic acid solution.
5. Thallos Stock: Add 60 μL of a 6.7% w/v pluronic acid solution into 100 μg of the cell-permeable Thallos-AM (VU053734–1) dye (TEFlabs cat # 0901sp, actual MW 841). Thallos-AM stock can be stored in aliquots at −20 °C for 1 month.
6. Dye Buffer: Add 6 μL of the Thallos-AM stock to 10 mL Assay Buffer. This volume is sufficient for one 384-well plate.
7. Thallium Sulfate Stock solution: Prepare 100 mM Thallium sulfate (Tl_2SO_4) solution by dissolving 2.52 g into 50 mL of ddH_2O. CAUTION: Thallium is extremely toxic and should be handled with care. Always wear gloves when handling thallium buffers and ensure proper waste disposal with local environmental health and safety representatives.

8. 5 × Thallium Working Solution: Add 0.2 mL of 100 mM Thallium Sulfate to 10 mL Assay Buffer for a final working solution of 2 mM Tl^+. This solution is a 5 concentrated stock that will be added during the assay.
9. Peptide Ligand Buffer: Assay Buffer containing 0.1% w/v Bovine Serum Albumin (BSA). Filter sterilize.
10. Small Molecule Ligand Buffer: Assay Buffer containing 0.1% DMSO.

2.2 Cell Culture Materials

1. Culture Media: 10% FBS supplemented DMEM containing Materials 4.5 g/L Glucose and 50 U/mL Penicillin-Streptomycin.
2. T-REx™ selection media (B-Media): Culture Media containing 0.5 mg/L Blastocidin.
3. Kir channel selection media (BH-Media): B-media containing 200 μg/mL Hygromycin.
4. Double Selection Media (BGH-Media): BH-Media containing 500 μg/mL Geneticin.
5. Plating media: 10% FBS supplemented DMEM containing4.5 g/L Glucose and 1 μg/mL Tetracycline–HCl.
6. Assay plates: Cell Plate: BD BioCoat Poly-D-Lysine 384-well small volume black microplate.

2.3 Robotics

1. FDSS plate reader with integrated liquid-handling head and kinetic fluorescence detector (Flo3).
2. 384-head automated liquid dispensing platform.
3. Automatic cell plate washer (Optional—*see* Note 1).

2.4 Ligands (Peptides)

1. All neuropeptides are diluted in Peptide Ligand Buffer at a (Peptides) 2 × final concentration.
2. All small molecules are diluted in Small Molecule Ligand Buffer a 2 × final concentration.
3. Compound Plate: Greiner 384-well polypropylene plate, V-bottom (cat. # 781280).

3 Methods

3.1 Generation of Cell Lines Expressing Different Kir Subunits and Melanocortin Receptors

3.1.1 Generation of Polyclonal Cell Lines

1. Tetracycline-Regulated Expression HEK293T cells (T-REx™) of Cell Lines expressing a tetracycline regulatory element are transfected (by Expressing Different using Lipofectamine 2000) with a plasmid containing the Kir Subunits and desired Kir coding sequence (Kir7.1, Kir4.2 etc.) in a Melanocortin pcDNA5/TO plasmid backbone (*see* Note 2).
2. Two days following transfection, a polyclonal population of Kir-expressing cells is selected using BH media.
3. After 30 days, stable Kir cells are then transfected with the desired GPCR (MC4R, MC3R, etc.) or a mock vector in a pcDNA3 backbone.
4. Two days following transfection, cells are selected for GPCR expression using BGH media.
5. Thirty days following this second selection, a Kir-GPCR polyclonal cell line is now ready for clonal selection.
6. If characterizing the presence of a Kir-GPCR interaction, polyclonal cell lines can be used. However, if a known Kir-GPCR interaction will be examined in a HTS format, selecting mono-clones using the process below is highly recommended.

3.1.2 Generation of Monoclonal Cell Lines (See Notes 3 and 4)

1. Single-cell clones are selected by diluting polyclonal cell lines to 0.8 cells/20 μL in BGH media and then plating them on a poly-D-lysine-coated 384-well culture plate.
2. Wells are examined for the presence of single-cell colonies over the next 30 days. Wells that contain more than one clone are noted and excluded.
3. BGH Media is added weekly until cells become confluent.
4. Once confluent, monoclone cells are transferred to progressively larger culture vessels to ensure sufficient cell number for clonal screening.
5. HEK293T monoclones stably expressing a Kir channel and a GPCR can now be screened for using the protocol described in Subheading 3.2.

3.2 Assay Protocol

3.2.1 Cell Plating

1. Grow cells in BGH media in a 150 mm cell culture dish until 70–90% confluent.
2. Wash cells with DMEM and then disassociate using 5 mL0.05% trypsin in DMEM.

3. Collect cells in a 15 mL conical tube and add 10 mL plating media.
4. Pellet cells in a swinging bucket centrifuge at 250 × *g* for 5 min.
5. Aspirate the supernatant and resuspend the cell pellet in 10 mL plating media containing Tetracycline to induce channel expression. Tetracycline concentration must be optimized empirically but we have found that 1 μg/mL is sufficient.
6. Count cells on a hemocytometer and dilute to 10^6 cells/mL with plating media containing Tetracycline. A final 75–90% confluency is optimum.
7. Pour the cell suspension into a sterile reservoir. Using an electronic multichannel repeat pipettor, immediately dispense 20 μL into each well of a black-wall, clear-bottom, 384-well poly-D-lysine-coated assay plate (BD Biosciences, Bedford, MA).
8. Lightly tap the corner of the plate to ensure that the cell suspension reaches the bottom of each well. Bubbles should be avoided as they prevent a uniform cell distribution.
9. Place the cell plate in a 37 °C 5% CO_2 incubator for 20–24 h to ensure cell adherence and protein (receptor and channel) expression.
10. If running an assay that requires 24-h incubation with a ligand, add compounds at this time.

3.2.2 Assay Procedure

1. 20–24 h following cell plating, check the assay plate to make sure that the cells are uniformly distributed within each well. If certain wells appear nonuniform, note them in a lab notebook so that they can be excluded from the final analysis.
2. Prior to beginning the assay, prepare all buffers described in Subheading 2.1.
3. Remove the plating media by flicking the plate. Remove any residual media by slamming the plate on a set of clean laboratory napkins (*see* Note 1).
4. Add 20 μL of Dye Buffer to each well using an electronic multichannel repeat pipettor (*see* Note 1).
5. Incubate cells in the dark for 30–60 min at room temperature. This incubation allows for intracellular esterases to cleave the acetoxymethyl-ester group from the Thallos-AM dye. This ensures intracellular accumulation of the dye and prevents dye efflux.
6. During this incubation, prepare the compound plate. Compound plates containing a ligand compound and the vehicle should be made in a checkerboard pattern (Fig. 1 and see Note 5). Prepare the ligands at 2 × the final desired concentration.
7. Once the incubation is complete, the dye buffer is removed by again flicking the plate and then slamming on a set of clean laboratory napkins. Then, add 20 μL of Assay Buffer to each well using an electronic multichannel repeat pipettor.

8. Using a 384-head automated liquid-handling robot, transfer 20 μL of ligand solution from the compound plate to the corresponding well in the assay plate. This step can be done inside the plate reader or on a separate machine depending on the specific assay design.

9. If using α-MSH, incubate the Assay plate for 20 min (Fig. 1 and *see* Note 6).

10. During this incubation, prepare a compound plate containing 25 μL of Thallium buffer in every well.

11. Following the ligand incubation, place the assay plate in a 384-well kinetic plate reader with liquid-handling capabilities. Record in the fluorescence mode with an excitation at 470 nm using an argon laser. Emission was filtered using a 530 ± 30 nm bandpass filter. The protocol is as follows:

    a. 12 min total recording time with sampling at 1–2 Hz.

    b. While recording, add 10 μL of 5 × thallium buffer into all wells using the 384-well liquid-handling module after 2 min of baseline recording.

3.3 Data Analysis

1. Data from individual assay wells are exported as normalized fluorescence responses (F_{ratio}), obtained by dividing each data point (F_{raw}) by the average of the fluorescence signal during the two-minute period of baseline recording (F_0) (Fig. 2a):

$$F_{\text{ratio}} = F_{\text{raw}} / F_0 .$$

2. Effects of a ligand on Kir channel opening ($F_{\text{Net\_Response}}$) are quantified and displayed by subtracting the fluorescence response of a well treated with the ligand ($F_{\text{ratio\_Compound}}$) from that of a well treated with the vehicle directly below or above ($F_{\text{ratio\_Vehicle}}$). This gives the net effect of a ligand on the channel opening ($F_{\text{Net\_Response}}$) and is also referred to as the "ratio subtracted" measurement (ΔF_{ratio}) (Fig. 2a, b):

$$F_{\text{Net\_Response}} = \Delta F_{\text{Ratio}} = F_{\text{Ratio(Compound)}} - F_{\text{Ratio(Vehicle)}} .$$

3. Once the ΔF_{Ratio} for a ligand has been determined, the maximum or minimum ΔF_{Ratio} ($\Delta F_{\text{Ratio Max}}$) of each replicate can be determined and used to statistically compare between groups.

4. *Z*-Factor, a measure of statistical effect size and variability, is used to investigate the high-throughput screening viability of an assay. A *Z*-factor > 0.5 is considered acceptable for high-throughput screening. The Z-factor can be calculated using the following formula:

$$Z\text{-factor} = 1 - \frac{3(\sigma_c + \sigma_v)}{|\mu_c - \mu_v|} .$$

μ_c = Average ΔF_{Ratio} Compound -Vehicle.

μ_v = Average ΔF_{Ratio} Vehicle -Vehicle.

σ_c = Standard Deviation ΔF_{Ratio} Compound Vehicle.

σ_v = Standard Deviation ΔF_{Ratio} Vehicle Vehicle.

3.4 Results

We have employed the thallium flux assay to characterize the MC4R-Kir7.1 signaling pathway in HEK293 cells stably expressing MC4R and Kir7.1-M125R. The use of the Kir7.1M125R mutation is critical as wild-type Kir7.1 has an extremely low single-channel conductance (~50 fS) which reduces the assay signal. As reported previously, a 20 min exposure of the MC4R agonist α-MSH reduces Kir7.1-mediated thallium flux by up to 20%, indicating decreases in channel opening (Fig. 3a). Investigating the viability of the α-MSH response for a high-throughput screening using a checkerboard plate format (Fig. 1) and the analysis method described in Subheading 3.3, an assay Z-Factor of 0.19 was obtained for 100 nM α-MSH (Fig. 3b). Under these given circumstances, this assay of MC4R-Kir7.1 signaling classifies as “medium throughput” assay.

In addition to studying the effects of α-MSH, we have also used the thallium flux assay to characterize the effects of the MC4R inverse agonist AgRP on MC4R-Kir7.1 signaling. Using a checkerboard format as depicted in Fig. 1, 100 nM AgRP was found to increase thallium flux relative to vehicle control indicating increases in channel opening (Fig. 4). Together as previously reported, these results indicate that the thallium assay can be employed to study molecular pathways involved in MC4R-Kir7.1 signaling.

Given the regulation of Kir7.1 by MC4R ligands, we sought to devise an experimental protocol to investigate the molecular signaling pathways that mediate this interaction. Previous electrophysiology experiments have shown that MC4R regulates Kir7.1 through a G-protein-independent manner. Using the strategy outlined in Fig. 5, we first examined a role of cAMP-dependent activation of Protein Kinase A (PKA) in this signaling pathway. After flicking off the Thallos-AM dye, 10 μL of a 3 × Rp-cAMP solution, a potent competitive inhibitor of the activation of cAMP-dependent protein kinases, was added to the cell plate. Following a 2 min incubation, 10 μL of a 4 × α-MSH buffer was then added to the assay plate. Using the analysis described in Subheading 3.3, we found that Rp-cAMP was unable to block MC4R-Kir7.1 signaling (Fig. 6a). Examining directly a role of $G_{s\alpha}$ next, we employed a dominant negative G_s construct that has been shown to completely block the canonical $G_{s\alpha}$-AC-cAMP signaling pathway. Co-expression of this construct in HEK293 cells was not able to affect EC_{50} of the α-MSH concentration response curve when compared to transfection with a control $G_{s\alpha}$ plasmid (Fig. 6b). Together, these results support our earlier findings [7] that the MC4R-Kir7.1 signaling is independent of G_s-AC-cAMP signaling pathway. Furthermore, these data suggest that a noncanonical GPCR signaling pathway may be essential for this interaction.

4 Notes

1. Removing the media and assay buffers in a consistent manner is critical for this assay. In the past we have used several expensive plate-washing robots for these purposes. However, the most consistent and cost-effective manner is the “flick and slam” method:
   a. Using your dominant hand, hold the plate with your palm facing (but not touching) the bottom of the plate.
   b. Next, bend your arm and wrist so that the top of the plate faces your chest.
   c. Finally, rapidly adduct your arm and wrist in a single-fluid movement to expel the buffer contained in the plate into a sink. Wash the media down the drain with 10% bleach.
   d. Finally, invert the plate onto a set of clean laboratory napkins and repeatedly slam the plate down until all of the remaining media is removed.
2. Stable cell lines are critical when performing the thallium assay to characterize GPCR-Kir interactions. Using a tetracycline regulatory system enables the generation of stable cell lines with constructs that may prove toxic when constitutively expressed. Channels that are not toxic when overexpressed may not require this system. However, if you are unfamiliar with the channel you are studying, it is pragmatic to choose this approach.
3. Generation of mono-clones is not necessary when characterizing the presence or absence of a GPCR-Kir interaction. We have successfully employed polyclonal cell lines in this regard. However, when attempting to run this assay in a medium to high-throughput manner, monoclones are essential. Clone selection should be determined empirically using the assay-screening format employed in all future screening.
4. In an attempt to create cell lines more rapidly, our group has used other ways to rapidly generate monoclones. Our more recent studies on MC4R-Kir7.1 signaling were performed on a cell line using the Flp-In/T-REx 293 system (Thermo). This cell line contains a single FRT site that enables FLP recombinase-mediated insertion of the pcDNA5/FRT/TO plasmid. This enables inducible expression of the vector contained in the ORF of the plasmid. Using this plasmid, we designed a bicistronic vector with hMC4R and hKir7.1 (M125R) potassium channel using the cleavable peptide linker p2A. This method required a single round of selection in BH media because recombination and transcription is only possible from the FRT locus.
5. Creating a compound plate is critical because it enables uniform addition to each well. In our experience, a minimum of 12 compound-treated wells and 12 vehicle-treated wells are necessary to observe changes in Tl^+ flux caused by melanocortin agonists. This is largely due to a small signal-to-noise ratio and

intrinsic assay variance. If possible, do not use Row A, B, O, and P as they are subject to an edge effect.

6. It may be necessary to optimize the incubation time for different ligands. This can be accomplished with a time course as depicted in Fig. 5.

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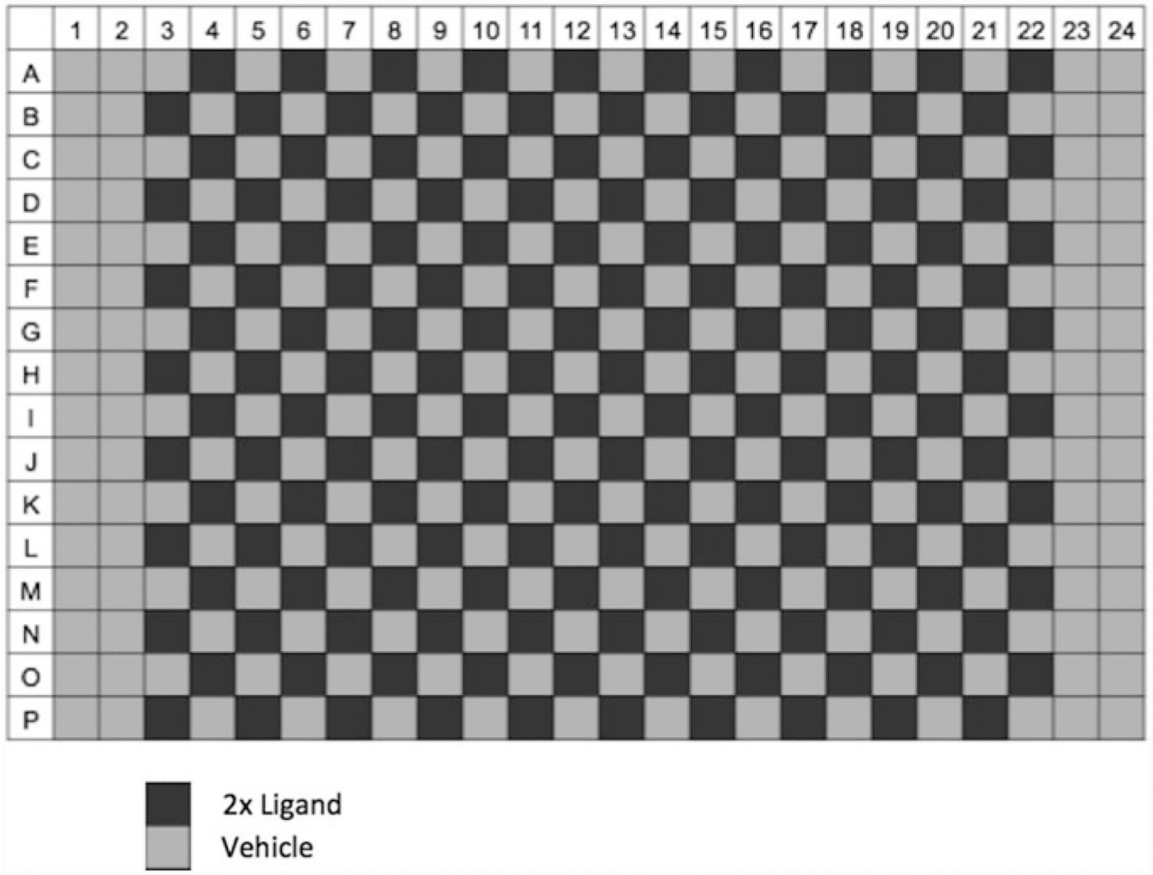

Fig. 1.

Sample plate map: calculation of the ΔF_{Ratio} necessitates the use of a checkerboard plate map. F_{Ratio} traces from agonist-treated well traces are subtracted from the vehicle-treated trace directly above (*odd columns*) or below (*even columns*) to obtain ΔF_{Ratio}. We have found that 12–16 compound well/vehicle subtractions, or two columns, are necessary to detect significant differences in Kir7.1 flux

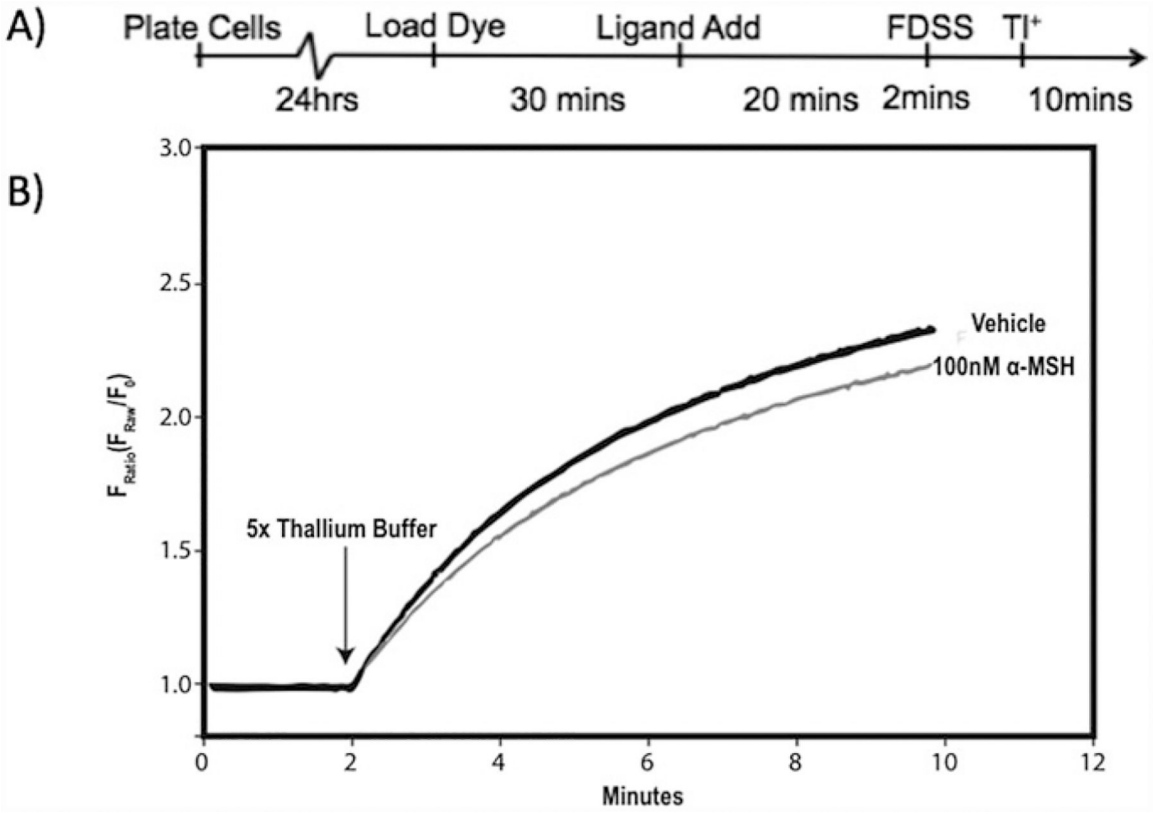

Fig. 2.

Experimental protocol and sample traces: (**a**) A cell plate is prepared with 2×10^4 cells/well in a 384-well plate and incubated for 24 h. Cells are then washed with assay buffer, loaded with dye buffer, and incubated for 30 min. After another wash with assay buffer, the ligand is added using a 384-well robotic pipettor to ensure that every well receives agonist at the same time. After 20 min, the plate is now ready to be read in the FDSS kinetic plate reader. (**b**) Once placed in the FDSS reader, a 2 min baseline reading (F_0) is obtained. Using the FDSS automated liquid handler, 10 μL of a 5 × Thallium buffer is added to each well of the assay plate. Fluorescence is read for 10 min following this addition. ΔF_{Ratio} is then calculated using the method described in Subheading 3.3

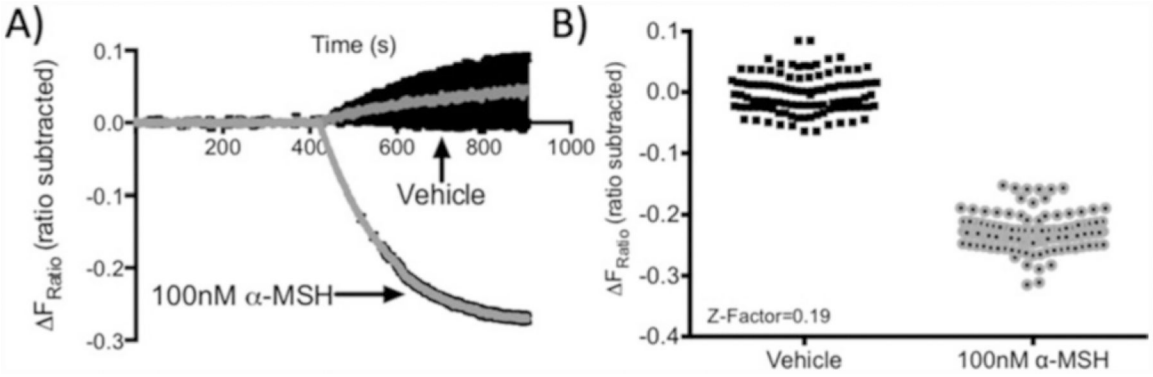

Fig. 3.

α-MSH reduces Kir7.1-mediated thallium flux: (**a**) HEK 293 T cells expressing MC4R and Kir7.1 plated in a 384-well plate are exposed to a 20 min incubation of 100 nM α-MSH in a checkerboard pattern. This agonist causes a reduction in ΔF_{Ratio} following the addition of thallium. Gray average of ΔF_{Ratio} measurements, Error bars ± SEM $n = 110$. (**b**) Using the ΔF_{Ratio} a Z-factor of 0.19 was throughput assay. $n = 110$

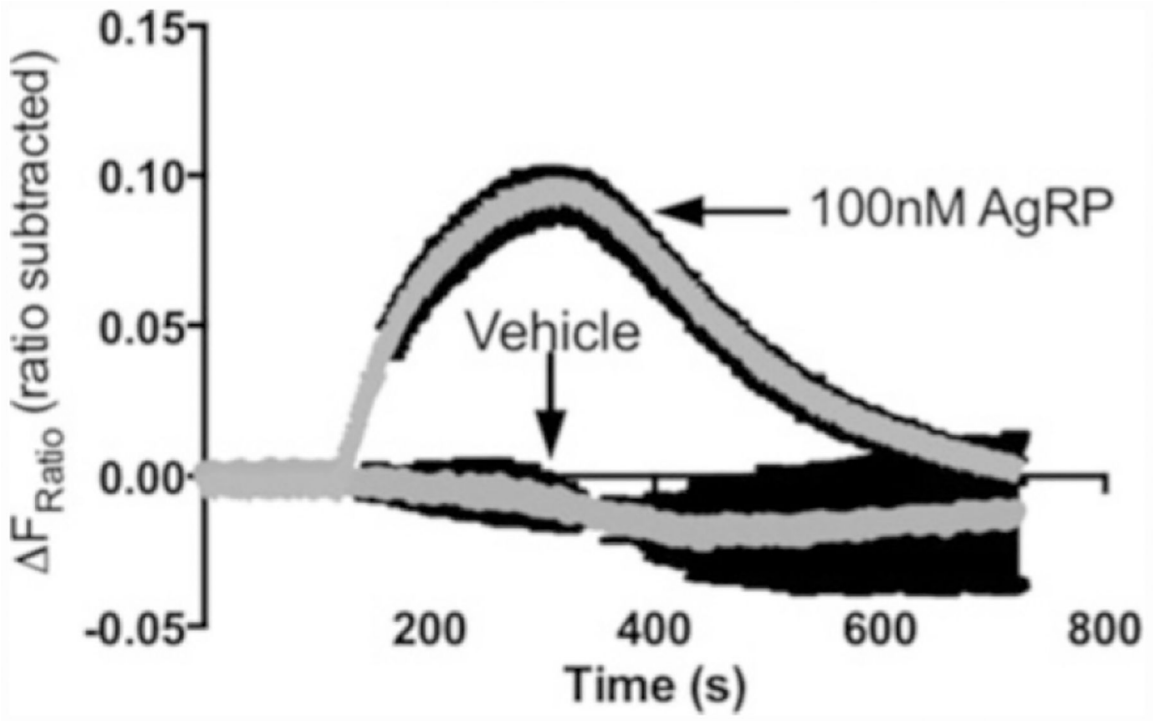

Fig. 4.

AgRP increases Kir7.1 mediated thallium flux: 100 nM AgRP increases thallium flux in HEK293 cells expressing MC4R and Kir7.1. Gray average of ΔF_{Ratio} measurements, Error bars ± SEM. $n = 36$

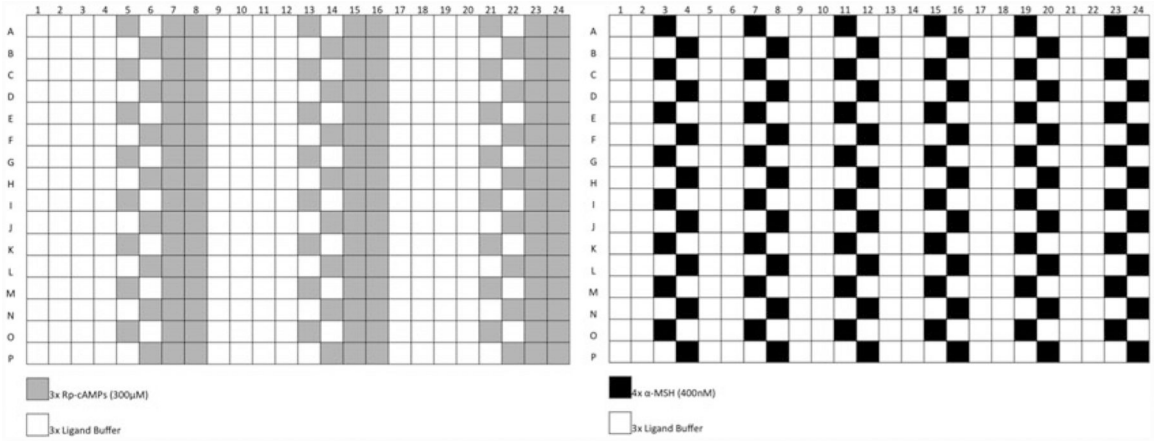

Fig. 5.

Plate map strategy for analyzing compounds that modulate MC4R-Kir7.1 signaling: This plate map format allows the study of pharmocological probes in the thallium flux assay. In this example, Rp-cAMPs, a potent competitive inhibitor of the activation of cAMP-dependent protein kinases, is dispensed in a checkerboard format on *columns* 5 and 6 but in every well of columns 7 and 8. Following a 10 μL addition of this compound to the assay plate, a second 10 μL stimulus of 400 nM (4 ×) α-MSH is then added. This plate is designed in a checkerboard format on *columns* 3 and 4 for a positive control and *columns* 7 and 8 to determine the ability of Rp-cAMPs to block the effect of α-MSH

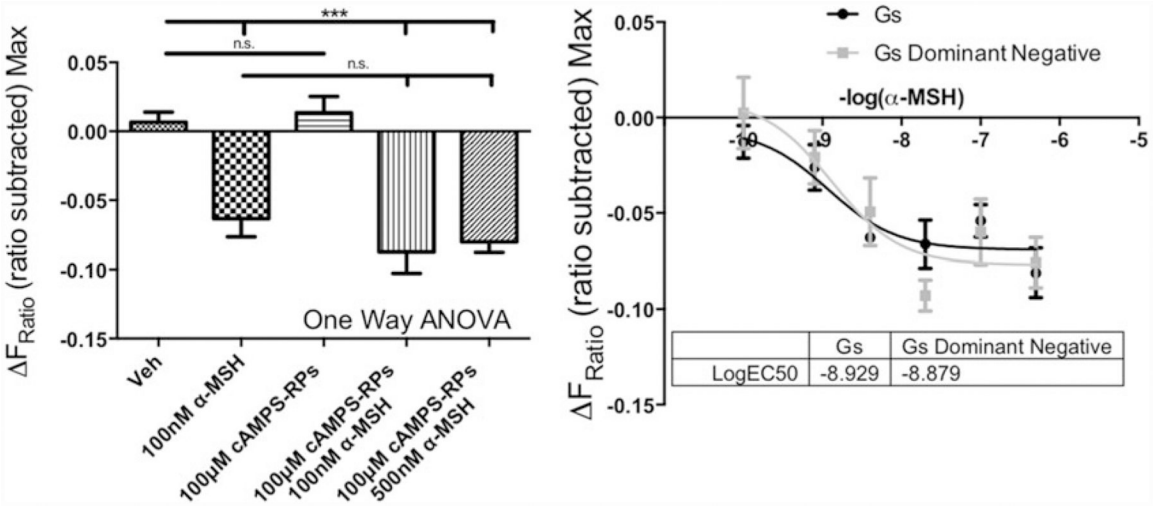

Fig. 6.

MC4R-Kir7.1 signaling is not regulated by Gs: (**a**) 100 μM Rp-cAMPs does not block the effect of α-MSH on Kir7.1 conductance. $n = 36$ ΔF_{Ratio} measurements/group; Error bars ± SEM; One Way ANOVA $p < 0.001$. (**b**) In this experiment, a dominant negative Gs plasmid or a wild-type Gs plasmid is transfected into HEK293 cells expressing MC4R and Kir7.1. Forty-eight hours later, cells are exposed to a dose response curve of α-MSH. Expression of a dominant negative Gs plasmid in MC4R-Kir7.1 cells does not right shift the α-MSH dose response curve as indicated by the lack of an effect on the EC_{50} of α-MSH. $n = 12$ ΔF_{Ratio} measurements/group; error bars ± SEM; One Way ANOVA $p < 0.001$